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Introduction

Breast cancer is the most common form of malignancy affecting women in developed countries. The growth of a majority of breast tumors is profoundly influenced by hormonal factors. Estrogen, the female sex steroid hormone, is a powerful mitogen and has been shown to induce proliferation of established breast cancer cells (1, 2). Estrogen exerts its effects through a nuclear ligand-activated transcription factor, the estrogen receptor (ER). An activated hormone-receptor complex transactivates genes that are responsive to the steroid hormone by binding to Estrogen Response Elements (EREs), specific DNA elements in the promoter region of the gene (3). Human breast tumors that are estrogen-responsive express a significantly higher amount of estrogen receptor (ER) compared to normal breast tissue and are designated as ER positive. Breast tumors that are ER negative usually are more aggressive, occur earlier in life, are resistant to endocrine treatment and have a poor prognosis and survival rate (4, 5). In addition, ER positive and negative breast tumors also exhibit distinct phenotypic difference (6).

Purpose of the present work

The research proposed in this grant proposal is based on the hypothesis that the involvement of estrogen in breast cancer proliferation and progression may be due to its selective regulation of a repertoire of genes by acting through ER and ER related factors. The experiments described and the results presented in this report address Task #1, Task #2a and Task #3a and 3b of this US Army Grant DAMD17-99-1-9384. Results from additional experiments performed in order to reinforce the hypotheses presented in the research proposal have also been included.

Key research accomplishments:

(1) GREB genes and submission to GenBank database (Task #1a, 1b):

Preliminary studies presented in the research grant proposal had demonstrated the success of Suppression Subtractive Hybridization (SSH) in isolating estrogen responsive genes that were differentially expressed in response to estrogen in MCF7, an ER+ breast carcinoma cell line. Approximately 400 putative subtracted clones were generated. Of these, 240 clones were screened using Northern blot analysis of mRNA from MCF7 cells grown in presence or in the absence of β-estradiol. Twenty-four cDNA clones were differentially expressed. Partial sequencing of the cDNA inserts revealed that several genes had been detected multiple times. To date, 14 different estrogen responsive genes have been isolated that have shown differential levels of expression ranging from 4 fold to on/off (fig. 1). Only 3 of these genes isolated through SSH matched known genes in

the GenBank/EMBL databases. However, none of the genes isolated through this study have been associated with hormone responsive breast cancer. The known genes include thrombospondin, PDZK1, and an immunoglobulin gene. The genes isolated in this study were designated as GREB (Genes Regulated by Estrogen in Breast cancer). The cDNA clones which were generated by SSH have been submitted to the GenBank/EMBL database and assigned the following accession numbers: GREB3, BE491961; GREB18, BE491962; GREB21, BE491963; GREB65, BE491964; GREB203, BE491965; GREB76, BE491966; GREB80, BE491967; GREB89, BE491968; GREB98, BE491969; GREB138, BE491970; GREB181, BE491971; GREB199, BE491972; GREB227, BE491973. The three different forms of GREB1, each having alternate 5′ and 3′ ends have been assigned the accession numbers AF245388, AF245389 and AF245390 for GREB1a, GREB1b and GREB1c, respectively.

(2) Effect of tamoxifen on expression of the 14 GREB genes

For more than two decades tamoxifen has been used to treat patients with an advanced stage of breast cancer. Tamoxifen acts as an inhibitor of estrogen-induced responses and also inhibits the effect of estradiol on cell proliferation and on regulation of specific genes. Additionally, in 1998, the US Food and Drug Administration (FDA) approved Nolvadex (tamoxifen citrate) for the reduction of the incidence of breast cancer in women at high risk of developing the disease (7). Therefore in keeping with our long-term goal of developing a clinical marker and also in devising novel therapeutic applications towards breast cancer treatment, it was necessary to ascertain the effect of tamoxifen on the expression of the 14 GREB genes. Northern blot analysis of MCF7 cells grown in the absence or in the presence of β-estradiol, and in the presence of β-estradiol and tamoxifen, indicated that all the 14 genes that were induced by β-estradiol were repressed by tamoxifen (fig.1). Expression of the estradiol-responsive gene, pS2, was also found to be repressed by tamoxifen as has been reported previously (8). This would indicate the possibility that all the estrogen responsive genes isolated in this study were under the control of estrogen receptor.

(3) Time course study of gene induction by β-estradiol

In order to study the kinetics of gene induction in response to estradiol stimulation, the expression of the GREB genes at different time points after addition of β-estradiol was examined. MCF7 cells grown in estrogen free media for five days were then supplemented with β-estradiol ($1x10^{-8}$ M) for 6, 24 or 48 hours before harvesting. A similar set of MCF7 cells grown in the absence of β-estradiol was used as a control. Northern blot analysis revealed that 13 of the 14 genes examined responded early to β-

estradiol treatment, as gene expression was apparent within 6 hours of treatment. These results suggest that these genes are direct targets of activated estrogen receptor. Gene expression was detected after 48 hours for the one gene that did not respond early. Figure 2 shows representative examples of early responders to the β-estradiol treatment.

(4) Examination of estrogen responsive gene expression on a panel of breast cancer cell lines (Task #1c).

At this stage, our goal was to focus efforts on detailed functional studies of selected genes whose pattern of expression correlated with ER expression in breast cancer cell lines. Also, we wanted to correlate estrogen-responsiveness of the genes to ER expression in human breast carcinomas. Therefore, we examined the expression of the 14 estrogen-responsive genes in a panel of breast cancer cell lines. The cDNA fragments isolated during SSH were used as probes on Northern blots containing six ER-positive and two ER-negative human breast carcinoma cell lines. Six of the 14 genes examined (PDZK1, GREB1, and clones # 3, 21, 76, and 138) were expressed in some or all the ER-positive breast carcinoma cell lines and absent in all the ER-negative breast carcinoma cell lines. Figure 3 shows the results of the two estrogen-responsive genes, GREB1 and PDZK1, with the best correlation to ER expression. The expression pattern of pS2 is shown for comparison.

(5) Focusing efforts towards functional study of two estrogen responsive genes.

As is indicated in Figure 3, GREB1 shows a better correlation with ER than is seen with pS2, a gene that is often used as a marker for hormone-responsive breast cancer. In addition to exhibiting an on/off response to β-estradiol stimulation, GREB1 was expressed in all five of the strongly ER-positive cell lines and in none of the two ER-negative breast carcinoma cell lines or in BT-20, which has low levels of ER. In addition, GREB1 is induced by estradiol in ECC1, an endometrial carcinoma cell line (data not shown). PDZK1, which has been associated with breast cancer but has not been related to hormone-responsiveness, was detected in three of the five ER-positive breast cancers cell lines as well as in BT-20. Since the expression pattern of these two genes had the best correlation with ER expression in this panel of cell lines, we chose to focus further experiments on these two genes.

(6) Cloning and sequencing of GREB1 gene (Task #3a, 3b)

PDZK1 was first isolated in a yeast two-hybrid screen designed to identify proteins interacting with MAP17—a membrane-associated protein involved in regulation of cell proliferation in human kidney (9). It has been sequenced and assigned the GenBank Ac# NM_002614. Partial sequence analysis of GREB1 did not match any of

the known records in DNA sequence databases. Therefore for further functional studies, the cloning of full length GREB1 gene was undertaken.

(a) Cloning GREB1 cDNA

The GREB1 cDNA clone isolated through SSH was used as a probe to screen a random primed MCF7 cDNA library. Six positive clones were isolated. Sequencing of the two largest clones revealed two cDNAs of 2792 and 2347 bases with different 5′ and 3′ ends (Fig. 4a). These were designated as GREB1a and GREB1b, respectively. Screening of an oligo(dT)-primed MCF7 cDNA library yielded a third clone, GREB1c which had a poly A tail and in addition had 5′, 3′ ends that were divergent from GREB1a and GREB1b (Fig. 4a). The three clones were sequenced on both strands and analyzed. Comparison of the sequences with the EMBL-GenBank databases using BLASTN and BLASTP DNA analysis programs did not yield any significant similarity with known sequences in the database.

During SSH, another differentially expressed gene had been isolated that was the same size as GREB1 and also showed a similar pattern of expression in the multicell line blots. This gene had been designated as GREB2. It was possible that GREB1 and GREB2 were the same gene. Partial sequencing of GREB2 revealed similarity with KIAA0575 from human brain (GenBank Ac# AB011147) which is a mRNA coding for a protein of unidentified function. Conversely, there was no similarity of GREB1 with any known human sequences in the EMBL/GenBank database. However recently, a mouse gene was reported that had homology to both GREB1 and GREB2. We were unable to obtain a cDNA clone from the lambda cDNA library that spanned the region between these two clones. Therefore RT-PCR was used to clone the region not represented by the lambda clones. Two separate PCR reactions were performed using primers in the 3' end of GREB1 and the 5' end of GREB2. Based on the size of the mouse gene, these PCR reactions generated fragments of the expected size. Both PCR products were cloned and sequenced to determine the region between GREB1 and GREB2 thus confirming these two clones are from the same mRNA.

(b) Comparison between different forms of GREB1 gene

The longest cDNA of GREB1a is shown in Figure 4a. Using a combination of our lambda clones, the GenBank sequence of AB011147, and the PCR generated region between the two, the longest cDNA of GREB1 gene was obtained (see Fig 4a). The GREB1a mRNA is 8482 bases long (not including the poly A+ tail) which agrees with the size obtained by Northern blot. This mRNA is predicted to encode a protein of 1949 amino acids, which is shown in Fig 4b. However, the alternate transcripts from this gene may encode smaller proteins with variable carboxyl termini. Comparison of GREB1a,

GREB1b and GREB1c sequences using the Clustal V sequence alignment program (27) revealed that the 5′ untranslated region was dissimilar for all three cDNAs until 264 bases upstream of the putative start site of the longest open reading frame (ORF) predicted by DNA Strider version 1.2 (28) as exhibited in figures 4a, 4b and 4c. GREB1b differed slightly in that it had a deletion of 13 bases prior to the beginning of the sequence that matched for all three of the cDNAs. Other shorter clones GREB1b(i), 1b(ii) and 1d with this same 5′ end did not contain this deletion (Fig. 4c). Sequence homology between GREB1a and GREB1b continues for 1346 nucleotides (indicated by ✣) downstream of the putative start site beyond which the predicted ORF of GREB1b continues for an additional 28 nucleotides. Divergence of sequence homology between GREB1a and GREB1c occurs after 1159 nucleotides (indicated by ◆). The predicted stop codon for the GREB1c ORF is 71 nucleotides beyond the sequence divergence from GREB1a. GREB1b and GREB1c had putative ORFs of 457 and 409 amino acids, respectively. In addition, both their 3′ untranslated sequences were different from each other and from that of GREB1a (Fig. 4d). Within the region that was homologous, GREB1c had a single nucleotide change from an A to a C as compared with GREB1a and GREB1b. On translation, this alteration would result in a conservative amino acid change from Asparagine (N) to Threonine (T) as indicated with a (●) in figure 4b.

(c) GREB1a, GREB1b and GREB1c are genuine transcripts

In order to establish that GREB1a, GREB1b and GREB1c were genuine transcripts and not chimeras, Northern blot analysis and RT-PCR was performed. In addition, GREB1d, a transcript of 1kb that was isolated during the cloning process was included for comparison. GREB1d had been sequenced from both ends and on analysis revealed that sequences at the 3′ end of this clone matched with a ribosomal RNA gene while the 5′ end was unique and matched GREB1b. This seemed to imply that GREB1d could be a chimera.

Northern blot analysis using GREB1a, GREB1b and GREB1c cDNAs as probes confirmed differential expression of these transcripts. However, Northern blot analysis using GREB1d showed hybridization to the expected 8 kb band as well as to a 4.2 kb band that was not differentially expressed with β-estradiol treatment (data not shown).

Additionally, RT-PCR was performed on the four clones using 5′ primers that were common to all four transcripts and 3′ primers that were designed against the unique 3′ region of each clone. As indicated in figure 5, a single band of the expected size could be seen for GREB1a, GREB1b and GREB1c in the control lanes (with cDNA from each clone) and also in the lanes containing RNA from MCF7 grown in the presence of β-estradiol that had been reverse transcribed. Conversely, for GREB1d, which was believed to be a chimera, no expression of the gene was visible in the lane with reverse

transcribed MCF7 mRNA + β-estradiol confirming it was chimeric. In all cases, gene amplification was not visible in the negative control lanes (MCF7 mRNA without reverse transcriptase: Fig. 5, lane 3 of each set). These data prove that the cDNAs of GREB1a, GREB1b and GREB1c represent the expression of different mRNAs with divergent 5′ untranslated exons.

(7) Evaluating relative expression of GREB1 and PDZK1 in primary breast tumors using RT-PCR (Task #2a).

In order to ascertain the biological significance of GREB1 and PDZK1 in hormone responsive breast cancer, their expression patterns were evaluated in primary breast cancers by RT-PCR. Primers for this experiment were designed using sequence information from the cloned GREB1 gene and from the published sequence of PDZK1 (GenBank Ac# NM_002614).

Primary human breast tumor tissue was collected fresh from mastectomy and biopsy specimens (kindly provided by Dr. Helen Feiner, Breast Cancer Resource, NYU Medical Center) and snap frozen in liquid nitrogen. Preliminary experiments were performed in the absence of reverse transcriptase to verify that the PCR amplification was due to reverse transcription of mRNA present in the tumor samples and not due to contamination with genomic DNA in the total RNA extract. Qualitative analysis of the expression of GREB1, PDZK1 and ER genes in the primary tumors is presented in Figure 7a. As the figure indicates, GREB1 and PDZK1 were predominantly present in ER-positive breast tumors with only a few of ER-negative tumors exhibiting gene expression above background levels. Semi-quantitative analysis was performed by determining signal intensity using phosphorimaging. Values were normalized against β-actin to determine relative expression of the different genes analyzed in this study. As indicated in Figure 7b, GREB1 expression was 3.5-fold greater in ER-positive compared to ER-negative breast tumors. Similarly PDZK1 gene expression was 19-fold greater in ER-positive breast tumors than in ER-negative breast tumors.

Discussion

The mitogenic effects of estrogen in breast cancer growth and proliferation is acknowledged but not well understood. The physiologic responses controlled by estrogen that lead to breast cancer are still a matter for conjecture. Progesterone receptor (PR) (10), pS2 (11), HSP27 (12) and TGFβ (13), are genes induced by estrogen in hormone-responsive breast cancer that have been extensively studied. Although several physiologic functions have been ascribed to these genes, the expression of the known estradiol-regulated genes fail to adequately explain the effects of estrogen in hormone-responsive tumors. For this reason, we have undertaken a study to identify additional

genes induced in hormone-responsive tumors in response to estradiol treatment. Specifically, we were interested in identifying genes that were induced by estradiol, repressed by tamoxifen and that demonstrated a high degree of correlation with ER expression in breast cancer cell lines and primary tumors. The initial experiments outlined in this research proposal specifically listed under Tasks 1a, 1b, 1c, Task #2a and Task #3a and 3b enabled us to identify two genes—PDZK1 and GREB1—which fulfill these criteria.

Our endeavor in the years 2 and 3 of this grant proposal is to determine the role of these two genes in hormone responsive breast cancer. Presently, efforts are being made to clone the full-length gene in the correct reading frame into different expression vectors namely, pcDNA 3.1 (Strategene, Inc) and pcMS-EGFP (Clontech Laboratories, Ltd.) for transfection into MCF7 cells. pcMS-EGFP is an expression vector that coexpresses a green fluorescent protein. Therefore the transfected cells expressing the protein of interest can be evaluated using FACS analysis. We intend to assess the effect of overexpression of GREB1 and PDZ1 on cell proliferation as well as their effect on different phases of cell cycle in MCF7 cells that are grown without estrogen compared to control MCF-7 cells transfected with vector alone. The premise of these studies will be to ascertain whether a physiological phenomenon that is estrogen dependent loses its dependence on the hormone when these genes are constitutively expressed. This would indicate that these proteins are major regulatory agents in hormone-responsive breast cancer.

Additionally, antibody will be prepared against the gene products of GREB1 and PDZK1. Western analysis will be performed to analyze whether the transfected breast cancer cells are making the proteins. Also the antibodies will be instrumental in determining the status of the genes in breast tumors using immunohistochemistry in lieu of the in situ hybridization experiments outlined in task #2b of the research proposal. This will be more efficient than the originally proposed in situ hybridization, which is fraught with difficulties of nonspecificity and high background.

PDZK1 contains four PDZ domains and could presumably interact with a number of proteins simultaneously thereby coordinating the interplay of multiple proteins. Therefore we intend to analyze PDZK1 function through a yeast two-hybrid system to test for protein-protein interactions in breast cancer. This would be extremely important in establishing its role in the physiologic response of breast tumors to estrogen.

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Annual Progress Report (1st year)
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Key research accomplishments:

(Specifically address experiments outlined in Task#1, #2a and #3a and 3b. Results from additional experiments performed in order to reinforce the hypotheses presented in the research proposal have also been included.)

- Partial sequencing of 14 Genes Regulated by Estrogen in Breast cancer (GREB) isolated through SSH and submission of the sequences into GenBank database.
- Effect of tamoxifen in the expression of the 14 estrogen responsive genes.
- Time course study of gene induction by β-estradiol
- Examination of estrogen responsive gene expression on a panel of breast cancer cell lines.
- Focusing efforts towards functional study of two estrogen responsive genes.
- Cloning and sequencing of GREB1 gene.
- Evaluating relative expression of GREB1 and PDZK1 in primary breast tumors using RT-PCR.

Reportable outcomes:

Manuscript accepted for publication in Cancer Research

Title: PDZK1 and GREB1 are Estrogen-Regulated Genes Expressed in Hormone-Responsive Breast Cancer

A manuscript presenting the research achievements and the results presented in the sections above have been submitted to "Cancer Research". After some revisions the manuscript has been accepted for publication. It is currently in press and is scheduled for publication in the November 15th 2000 issue. The manuscript has been attached with this report along with the figures and figure legends, which also correspond to this progress report.

Attached manuscript:

A copy of the manuscript has been attached with this report along with the figures and figure legends, which also correspond to this progress report.

PDZK1 and GREB1 are Estrogen-Regulated Genes Expressed in Hormone-Responsive Breast Cancer

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Note: The nucleotide sequence data published in this paper has been submitted to the GenBank/EMBL database at NCBI and assigned the accession numbers AF245388, AF245389 and AF245390 for GREB1a, GREB1b and GREB1c, respectively. The cDNA clones which were generated by SSH have been submitted and assigned the following accession numbers: GREB3, BE491961; GREB18, BE491962; GREB21, BE491963; GREB65, BE491964; GREB203, BE491965; GREB76, BE491966; GREB80, BE491967; GREB89, BE491968; GREB98, BE491969; GREB138, BE491970; GREB181, BE491971; GREB199, BE491972; GREB227, BE491973.

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Abbreviations: ER, estrogen receptor; GREB, Genes Regulated by Estrogen in Breast cancer; PR, progesterone receptor; RT-PCR, Reverse Transcriptase-Polymerase Chain Reaction.

ABSTRACT

The function of estrogen in breast cancer proliferation and progression is likely to be due to the expression of a repertoire of genes regulated by estrogen receptor (ER).

Using suppression subtractive hybridization (SSH) we have isolated a set of 14 estrogen-responsive genes that was differentially expressed in MCF7 cells stimulated by β-estradiol as compared to unstimulated cells. Tamoxifen repressed the expression of all 14 estrogen-responsive genes. Thirteen of the genes were induced within 6 hours of estrogen treatment indicating that these were early response genes in the ER regulated pathway. PDZK1 and a new gene, GREB1, demonstrated a significant correlation with ER phenotype in a panel of breast cancer cell lines. Treatment with cycloheximide indicated that ER directly controls GREB1 expression. Three cDNAs (GREB1a, b and c) were isolated by screening a MCF7 cDNA library. These three cDNAs of GREB1 shared extensive sequences through the open reading frame but had divergent 5′ untranslated regions, indicating the possibility of multiple promoters regulated by β-estradiol. Studies in primary breast cancers showed that the expression of PDZK1 and GREB1 were over-expressed in ER-positive compared to ER-negative breast cancers by 19-fold and 3.5-fold, respectively. GREB 1 was also induced by β-estradiol in the ER-positive endometrial cell line ECC-1. The pattern of expression suggests a critical role for these two genes in the response of tissues and tumors to β-estradiol.

INTRODUCTION

Increased exposure to estrogen has been correlated with the incidence of breast cancer (1, 2). There is an increased incidence of breast cancer associated with younger age at menarche, late age at first pregnancy and late age of menopause. All these factors are associated with the duration of the exposure of breast tissue to estrogen (3, 4). In addition, clinical and laboratory studies have demonstrated that breast epithelial cells proliferate in response to estrogen (5). Therefore, it has been postulated that the mitogenic effect of estrogen, combined with inadequate DNA repair, may lead to an accumulation of genetic aberrations culminating in cancer (2). Additional evidence that estrogen is a major adverse factor in breast cancer progression is the observation of regression in tumor growth after ovariectomy and following treatment with antiestrogens like tamoxifen (reviewed in 6).

Estrogen exerts its effects through a nuclear ligand-activated transcription factor, the estrogen receptor (ER). An activated hormone-receptor complex transactivates genes by binding to regulatory promoter elements called Estrogen Response Elements (EREs) (7). Two known subtypes of ER exist, ERα and ERβ, with distinct tissue and cell patterns of expressions (8). In this study, ER specifically refers to the ERα. ER expression is used routinely as a clinical marker to predict the hormone responsiveness of breast tumors(9). Compared to ER-negative breast tumors, a high percentage of ER-positive tumors respond favorably to endocrine treatment, are associated with a better prognosis and have a well-differentiated phenotype (10-12).

The marked physiologic and phenotypic differences between the ER-positive and ER-negative breast tumors have been hypothesized to be due to differences in gene expression between these two tumor types. Studies have demonstrated the differential expression of a number of genes when comparing ER-positive and ER-negative breast carcinoma cell lines (2, 13-16). Although most of the genes found to be differentially expressed in ER-positive cancers were not estradiol-responsive, it is likely that estrogen induces the expression of a repertoire of genes controlling cell growth. Using techniques to

isolate differentially expressed genes, several estradiol-inducible mRNAs have been identified in ER-positive human breast cancer cell lines. Genes known to be induced by estrogen treatment include progesterone receptor (PR) (9), pS2 (17), cathepsin D (18), heat shock protein 27,000 (HSP27) (19), aldolase A, dehydrogenase, α-tubulin and glyceraldehyde-3-phosphate (reviewed in 20). In addition to these genes, a number of estrogen-induced mRNAs of unknown function have been isolated (2, 15).

The repertoire of genes responsible for the physiologic effects of estrogen on hormone-responsive breast cancers has not been completely characterized (2). In addition, well-characterized genes such as pS2 are only expressed in 30- 60% of hormone-responsive tumors (21-23) suggesting that its function is not necessary for normal physiologic response to estrogen. Therefore, in order to more completely understand hormone-responsive breast cancer, there is a pressing need to identify more estrogen-responsive genes and establish their role in tumorigenesis and progression.

In this study, efforts were made to delineate genes that are differentially expressed in response to estradiol treatment in MCF7, an ER-positive, hormone-responsive human breast carcinoma cell line. Using suppression subtractive hybridization (SSH) (24), a set of estrogen-responsive genes have been isolated that are also repressed by tamoxifen. Further screening has enabled us to focus on two estrogen-responsive genes that exhibit a distinct correlation with ER expression in breast carcinoma cell lines and primary breast carcinomas. The pattern of expression indicates a critical functional role of these genes in hormone-responsive tissues and cancer.

MATERIALS AND METHODS

Oligonucleotides

Oligonucleotides were obtained from Operon Technologies, Inc. (Alameda, CA).

Cell Lines

All cell lines were obtained from American Type Culture Collection (ATCC; Rockville, MD). Cell lines MCF7, T-47D, BT-20, MDA-MB-231, and HBL-100 were maintained in Minimal Essential Medium (Gibco BRL, Gaithersburg, MD); ZR-75-1 and BT-474 were maintained in RPMI 1640 (Gibco BRL); MDA-MB-361 was maintained in Leibovitz's L-15 medium (Gibco-BRL). Media were supplemented with 10% fetal calf serum (Gemini Bio-Products, Calabasas, CA), 10 U/ml penicillin G (Gibco BRL), 10 μg/ml streptomycin (Gibco BRL), and 6 ng/ml bovine insulin (Sigma Chemical Company, St. Louis, MO). All cells were incubated at 37°C in 5% CO_2 except MDA-MB-361 which were maintained in a CO_2-free environment.

MCF7 cells that were induced with estradiol were grown under normal conditions as described above until approximately 25% confluence. The media was subsequently changed to phenol-red free Minimal Essential Media (Gibco BRL) supplemented with 10% charcoal-stripped fetal calf serum (Gemini Bio-Products; certified to be β-estradiol-free), 10 U/ml penicillin G, 10 μg/ml streptomycin, 6 ng/ml bovine insulin. MCF7 cells were treated with $1x10^{-8}$ M water-soluble β-estradiol (Sigma Chemical Company) and/or $1x10^{-6}$ M 4-hydroxytamoxifen (Sigma Chemical Company) for periods of 6 -72 hours. Cycloheximide (Sigma Chemical Company) was used at a concentration of 10 μg/ml.

mRNA Isolation

Polyadenylated RNA was isolated from cell lines using the Fast Track® Kit 2.0 (Invitrogen® Corp., Carlsbad, CA) according to the manufacturer's instructions.

Suppression Subtractive Hybridization

Suppression Subtractive Hybridization (SSH) (24) was performed with the CLONTECH PCR-Select™ cDNA Subtraction Kit (CLONTECH Laboratories Inc., Palo Alto, CA) as described by the manufacturer. Starting material consisted of 2 μg of poly(A)$^+$RNA from MCF7 cells grown in the absence of β-estradiol for 5 days and then treated with β-estradiol for 72 hours (referred to as tester) and 2 μg of poly(A)$^+$RNA from MCF7 grown in the absence of β-estradiol for 8 days (referred to as driver). All PCR products generated using SSH were subcloned into the pCR™2.1 vector using the Original TA Cloning® Kit (Invitrogen®).

Isolation of GREB1 cDNAs

Approximately 1x10^6 plaques from an oligo-dT and random primed MCF7 cDNA library were screened using the 600 bp GREB1 fragment isolated by SSH. Lambda phage plaques were grown in *Escherichia coli* XL1-blue MRF' cells (Stratagene) and phage were transferred to Optitran™ supported nitrocellulose (Schleicher & Schuell) using the *in situ* plaque hybridization technique (25). Phage lifts were pre-hybridized and hybridized in a solution containing 50% formamide (v/v), 5x SSPE, 5x Denhardts, 0.1% SDS (v/v) and 100 μg/ml denatured salmon sperm DNA at 42°C. Fifty ng of GREB1 cDNA was ^{32}P-labeled by random priming (Roche Biochemicals) using 50 μCi of [α^{32}P]dCTP; 3000 Ci/mmole (Amersham). Following a 16 hour hybridization, membranes were washed in 2x SSC/0.1% SDS (v/v) for 20 min at 42°C, and twice in 0.2x SSC/0.1% SDS (v/v) for 20 min at 65°C prior to exposure to Kodak XAR-2 film, overnight at -80°C with intensifying screens. Lambda phage plaques that hybridized to ^{32}P-labeled 600 bp

GREB1 fragment were isolated and plaque purified by secondary and tertiary rounds of screening. Isolated plaques were processed to excise the cDNA inserts from the lambda vector to yield phagemids containing the inserts of interest in the pBK-CMV vector (Stratagene).

The region of GREB1 cDNA that was not obtained from screening the lambda library was obtained by RT-PCR. The region between GREB1 and GREB2 (later determined to be the 3' end of GREB1 cDNA) was amplified using two separate RT-PCR amplifications. One reaction used the primers 5'GREB1/2: (5'-TGGACCTGGGATCCTTTGAGAAGGTGGACTTTC-3') and 3'GREB1/2: (5'-ACGACGAGGTGACCGACACCTGGACGCTCCTCTG-3') which generated a DNA fragment of 1565 bp. The second reaction used the primers ck5'GREB1/2: (5'-CCCCAACATTGTGACACTTCACGTGACC-3') and ck3'Greb1/2: (5'-CCAAGCAGGAGGAGTTTATCAATCGCAGG-3') which generated a DNA fragment of 720 bp. Both PCR reactions were cloned and had the identical sequence for the intervening region between GREB1 and GREB2.

Northern Analysis

One μg of mRNA was electrophoresed on a 1% agarose formaldehyde denaturing gel in 1x MOPS and then transferred to a Nytran® membrane (Schleicher & Schuell). Approximately 25-50 ng of each clone in pCR™ 2.1 or pBK-CMV was ^{32}P-labeled by random priming (Roche Biochemicals). As controls, 25-50 ng cDNA for pS2, ER, GAPDH and β-actin were also labeled. Northern blots were pre-hybridized and hybridized in 50% formamide, 5x Denhardts, 5x SSPE, 1% SDS and 100 μg/ml denatured salmon sperm DNA at 42°C. Blots were washed in 2x SSC and 0.1% SDS at 42°C for 20 minutes followed by two washes in 0.2x SSC and 0.1% SDS at 65°C for 20 minutes each. Northern blots were placed on film with an intensifying screen at -80°C.

DNA Sequence Analysis

Sequencing of the SSH fragments was performed on double-stranded templates using the dideoxynucleotide chain-termination method with [α-^{35}S]dATP, 1000 Ci/mmol (Amersham) as label. Sequencing reactions were carried out using the Sequenase™ version 2.0 T7 DNA polymerase DNA Sequencing Kits (Amersham/USB, Cleveland, OH) with T7 promoter primer in the pCR™ 2.1 vector.

The sequence for the GREB1a, GREB1b and GREB1c cDNAs isolated from the MCF7 cDNA library was determined on both strands using the automated ABI 373 DNA sequencing system with the standard dye terminator AmpliTaq polymerase FS kit, by the Stanford University PAN facility (Stanford, CA). T3 and T7 promoter primers, and custom sequence specific primers were used, and all sequence reported was determined by sequencing both strands of DNA. The nucleotide sequence of the cDNA and protein sequence of GREB1a, GREB1b and GREB1c were compared against the NCBI databases using BLASTN and BLASTP, respectively.

RT-PCR from Primary Tumors

Primary human breast tumor tissue was collected fresh from mastectomy and biopsy specimens (kindly provided by Dr. Helen Feiner, Breast Cancer Resource, NYU Medical Center) and snap frozen in liquid nitrogen. Approximately 0.5 mg of tissue was homogenized and total RNA was isolated using TRIzol® reagent (Gibco BRL) as per the manufacturer's recommendations. One μg of RNA from each sample was reverse transcribed using random hexamers with the Advantage™ RT-for-PCR kit (Clontech) as per the manufacturer's recommendations. The 20 μL RT sample was diluted to 100 μL with water and then 2 μL of each sample was analyzed by PCR for GREB1a, GREB1b and GREB1c,

PDZK1, ER and β-actin messages using the Advantage™ cDNA PCR kit (Clontech) with Advantage™ KlenTaq Polymerase mix and gene specific primers designed across intron/exon junctions. The common 5' primer for GREB1a, GREB1b and GREB1c was oGREB5' (5'-GGACCAGCTTCAGTCACCTTTCCAGTGGTGGCC-3'). The 3' primer for GREB1a was oGREB1a/3' (5'-GGAAGATCTGCTCCAGGTCCTCCATCAAGGGC-3'), for GREB1b was oGREB1b/3' (5'-CTCTTTTATGAATTGTCTGGTTTATACGTCCGG-3') and for GREB1c was oGREB1c/3' (5'-GGGAGTAAAGCTGGTGCCTGGGGCACAGGTCA CG-3'). The fragments generated for GREB1a, GREB1b and GREB1c, were 334, 339 and 325 bp respectively. Primers for PDZK1 were oPDZ-5'RT (5'-GCTTCTCCTGCGAATTGAGAAGGACACCGAGG-3') and oPDZ-3'RT (5'-GGTCCAAGTTTGCACACC TCCATTCATCACA GG -3') which generated a PCR fragment of 331 bp. Primers for ER were oER-1A-long (5'-GTGCCCTACTACCTGGAGAACGAGCCCAGC-3') and oER-1B-long (5'-AGCATAGTCATTGCACACTGCACAGTAGCG-3') which generated a 195 bp DNA fragment. Primers for β-actin were oACTIN-5' (5'-AGCAAGAGAGGCATCCTCACCCTGAAGTACC-3') and oACTIN-3' (5'-CAGATTCTCCTTAATGTCACGCACGATTTCCC-3') which generated a 471 bp DNA fragment. Two-step PCR was utilized to amplify the genes and was performed on a Perkin Elmer 9600 DNA thermal cycler as follows: One cycle of 94°C for one minute; then 30 cycles of 94°C for 30 seconds and 68°C for 3 minutes; followed by one cycle of 68°C for 3 minutes. PCR samples were then analyzed for GREB1a, GREB1b, GREB1c, PDZK1, ER and Actin by electrophoresis on 1.5% agarose gels.

Following electrophoresis the samples were transferred onto 0.2 μm Nytran™ (Schleicher & Schuell) nylon membranes by Southern blotting. Blots were pre-hybridized and hybridized in 20% formamide, 5x Denhardts, 5x SSPE, 1% SDS (v/v) and 100 μg/ml denatured salmon sperm DNA at 42°C. Southern blots were probed with oligonucleotides that had been end-labeled using [γ-^{32}P] ATP; 6000 Ci/mmole (Amersham) and T4 polynucleotide kinase (New England BioLabs). The oligonucleotides were designed internal to the sequence of each gene amplified. The four GREB transcripts were detected using oGREBint (5'-GGCTTGGCCTTGCATATTTTCAGC-3'). Other internal oligonucleotides were oPDZK1/int (5'-CATATGCAGGTTGTGGATCTGG-3'), oERpro-86 (5'-ACCCTGGCGTCGATTAT CTGAATTTGGC-3') and oActin-int (5'-ATGACCCAGATCATGTTTGAGACC-3'). Following 16 hours of hybridization, the blots were washed sequentially in 2x SSC/0.1% SDS (v/v) at 42°C for 20 minutes followed by two washes in 2x SSC/0.1% SDS (v/v) at 65°C for 20 minutes each and then were placed on X-ray film with an intensifying screen at for 10 minutes. Subsequently, the blots were placed on a Kodak Phosphor screen (Molecular Dynamics, Sunnyvale, CA) and quantified using ImageQuant software and a Molecular Dynamics Phosphorimager.

RESULTS

Isolating Estrogen Responsive Genes with SSH

SSH performed using mRNA from MCF7 cells grown in the presence and absence of β-estradiol, as tester and driver, respectively, resulted in the generation of more than 400 putative subtracted clones. Of these, 240 clones were screened using Northern blot analysis of MCF7 cells grown in presence or in the absence of β-estradiol. Through this process, 15 estrogen-responsive genes were identified. The difference in levels of expression ranged from approximately four-fold to on/off. The cDNA clones identified as differentially expressed were compared against the EMBL/GenBank databases. Only four of these clones had sequences that matched previously reported genes, however, none of these were known to be estrogen-responsive in breast cancer. These known genes include thrombospondin (GenBank Ac# X14787), PDZK1 (GenBank Ac# AF012281), KIAA0575 from human brain (GenBank Ac# AB011147) which is an mRNA coding for a protein of unidentified function, and an immunoglobulin like gene (GenBank Ac# A034198). The remaining 11 genes were deemed novel as their sequence did not match any of gene sequences previously reported. The novel genes as well as the known genes with undefined function have been designated as GREB (Genes Regulated by Estrogen in Breast cancer).

Effect of Tamoxifen on estrogen-responsive genes

Tamoxifen is a competitive antagonist of endogenous and exogenous estradiol. Tamoxifen acts as an inhibitor of estrogen-induced responses and also inhibits the effect of estradiol on cell proliferation and on regulation of specific genes. Thus, the effect of tamoxifen on the 15 estrogen-responsive genes was examined. Northern blot analysis of MCF7 cells grown in the absence or in the presence of β-estradiol, and in the presence of β-estradiol and tamoxifen, indicated that all the 15 genes that were induced by β-estradiol were repressed by tamoxifen (Figure 1). Expression of the estradiol-responsive gene, pS2, was also found to be repressed by tamoxifen as has been reported previously (26).

In uterus and bone, tamoxifen is known to be a partial agonist of estradiol. Therefore, we investigated whether tamoxifen treatment could upregulate genes in breast cancer cells compared to estrogen deprivation. SSH was performed using mRNA from MCF7 cells grown under strict estrogen deprivation as the driver while the tester sample comprised mRNA from MCF7 cells grown in the presence of β-estradiol and tamoxifen. Using Northern blot analysis, 126 putative subtracted clones were examined. However, none of the clones analyzed were found to be significantly upregulated by tamoxifen (data not shown). This result is consistent with the hypothesis that tamoxifen acts as an anti-estrogen in breast tissue and that the pattern of expression resulting from estrogen withdrawal is identical to the pattern of expression induced by tamoxifen treatment.

Time course study of gene induction by β-estradiol

In order to study the temporal response to estrogen stimulation, a kinetic study of gene induction of the 15 estrogen-responsive genes was undertaken in which the expression of the genes at different time points after addition of β-estradiol was examined. MCF7 cells grown in estrogen free media for five days were then supplemented with β-estradiol ($1x10^{-8}$ M) for 6, 24 or 48 hours before harvesting. A similar set of MCF7 cells grown in the absence of β-estradiol was used as a control. Northern blot analysis revealed that 14 of the 15 genes examined responded early to β-estradiol treatment, as gene expression was apparent within 6 hours of treatment. These results suggest that these genes are direct targets of activated estrogen receptor. Gene expression was detected after 48 hours for the one gene that did not respond early. Figure 2 shows representative examples of early responders to the β-estradiol treatment.

GREB1 and GREB2 are the same gene

The pattern of expression and size of the mRNAs suggested that GREB1 and GREB2 might be the same gene. As noted above, the sequence of GREB2 matched an

EST, KIAA0575 from human brain (GenBank Ac# AB011147), whereas GREB1 had no match in GenBank. More recently a mouse gene was reported that had homology to both GREB1 and GREB2. We were unable to obtain a cDNA clone from the lambda cDNA library that spanned the region between these two clones (see below). Therefore RT-PCR was used to clone the region not represented by the lambda clones. Two separate PCR reactions were performed using primers in the 3' end of GREB1 and the 5' end of GREB2. Based on the size of the mouse gene, these PCR reactions generated fragments of the expected size. Both PCR products were cloned and sequenced to determine the region between GREB1 and GREB2 thus confirming these two clones are from the same mRNA.

Expression of estrogen-responsive genes on a panel of breast carcinoma cell lines

We wanted to focus attention on genes whose pattern of expression correlated with ER expression in breast cancer cell lines. In order to correlate estrogen-responsiveness of the genes to ER expression in human breast carcinomas, we examined the expression of the 14 estrogen-responsive genes in a panel of breast cancer cell lines. The cDNA fragments isolated during SSH were used as probes on Northern blots containing six ER-positive and two ER-negative human breast carcinoma cell lines. Six of the 14 genes examined (PDZK1, GREB1, and clones # 3, 21, 76, and 138) were expressed in some or all the ER-positive breast carcinoma cell lines and absent in all the ER-negative breast carcinoma cell lines. Figure 3 shows the results of the two estrogen-responsive genes, GREB1 and PDZK1, with the best correlation to ER expression. The expression pattern of pS2 is shown for comparison. In addition to exhibiting an on/off response to β-estradiol stimulation, GREB1 was expressed in all five of the strongly ER-positive cell lines and in none of the two ER-negative breast carcinoma cell lines or in BT-20, which has low levels of ER. In addition, GREB1 is induced by estradiol in ECC1, an endometrial carcinoma cell line (data not shown). As is indicated in Figure 3, GREB1 shows a better correlation with ER than is seen with pS2, a gene that is often used as a marker for hormone-responsive

breast cancer. PDZK1, which has been associated with breast cancer but has not been related to hormone-responsiveness, was detected in three of the five ER-positive breast cancers cell lines as well as in BT-20. Since the expression pattern of these two genes had the best correlation with ER expression in this panel of cell lines, we chose to focus further experiments on these two genes.

Cloning GREB1 cDNA

The 600 base pair GREB1 cDNA clone isolated through SSH was used as a probe to screen a random primed MCF7 cDNA library. Six positive clones were isolated. Sequencing of the two largest clones revealed two cDNAs of 2792 and 2347 bases with different 5′ and 3′ ends (Fig. 4a). These were designated as GREB1a and GREB1b, respectively. Screening of an oligo(dT)-primed MCF7 cDNA library yielded a third clone, GREB1c which had a poly A tail and in addition had 5′, 3′ ends that were divergent from GREB1a and GREB1b (Fig. 4a). The three clones were sequenced on both strands and analyzed. Comparison of the sequences with the EMBL-GenBank databases using BLASTN and BLASTP DNA analysis programs did not yield any significant similarity with known sequences in the database.

The longest cDNA of GREB1a is shown in Figure 4a. Using a combination of our lambda clones, the GenBank sequence of AB011147, and the PCR generated region between the two, the longest cDNA of GREB1 gene was obtained (see Fig 4a). The GREB1a mRNA is 8482 bases long (not including the poly A+ tail) which agrees with the size obtained by Northern blot. This mRNA is predicted to encode a protein of 1949 amino acids, which is shown in Fig 4b. However, the alternate transcripts from this gene may encode smaller proteins with variable carboxyl termini. Comparison of GREB1a, GREB1b and GREB1c sequences using the Clustal V sequence alignment program (27) revealed that the 5′ untranslated region was dissimilar for all three cDNAs until 264 bases upstream of the putative start site of the longest open reading frame (ORF) predicted by DNA Strider

version 1.2 (28) as exhibited in figures 4a, 4b and 4c. GREB1b differed slightly in that it had a deletion of 13 bases prior to the beginning of the sequence that matched for all three of the cDNAs. Other shorter clones GREB1b(i), 1b(ii) and 1d with this same 5′ end did not contain this deletion (Fig. 4c). Sequence homology between GREB1a and GREB1b continues for 1346 nucleotides (indicated by ✣) downstream of the putative start site beyond which the predicted ORF of GREB1b continues for an additional 28 nucleotides. Divergence of sequence homology between GREB1a and GREB1c occurs after 1159 nucleotides (indicated by ◆). The predicted stop codon for the GREB1c ORF is 71 nucleotides beyond the sequence divergence from GREB1a. GREB1b and GREB1c had putative ORFs of 457 and 409 amino acids, respectively. In addition, both their 3′ untranslated sequences were different from each other and from that of GREB1a (Fig. 4d). Within the region that was homologous, GREB1c had a single nucleotide change from an A to a C as compared with GREB1a and GREB1b. On translation, this alteration would result in a conservative amino acid change from Asparagine (N) to Threonine (T) as indicated with a (●) in figure 4b.

In order to establish that GREB1a, GREB1b and GREB1c were genuine transcripts and not chimeras, Northern blot analysis and RT-PCR was performed. In addition, GREB1d, a transcript of 1kb that was isolated during the cloning process was included for comparison. GREB1d had been sequenced from both ends and on analysis revealed that sequences at the 3′ end of this clone matched with a ribosomal RNA gene while the 5′ end was unique and matched GREB1b. This seemed to imply that GREB1d could be a chimera. Northern blot analysis using GREB1a, GREB1b and GREB1c cDNAs as probes confirmed differential expression of these transcripts. However, Northern blot analysis using GREB1d showed hybridization to the expected 8 kb band as well as to a 4.2 kb band that was not differentially expressed with β-estradiol treatment (data not shown). Additionally, RT-PCR was performed on the four clones using 5′ primers that were common to all four transcripts and 3′ primers that were designed against the unique 3′

region of each clone. As indicated in figure 5, a single band of the expected size could be seen for GREB1a, GREB1b and GREB1c in the control lanes (with cDNA from each clone) and also in the lanes containing RNA from MCF7 grown in the presence of β-estradiol that had been reverse transcribed. Conversely, for GREB1d, which was believed to be a chimera, no expression of the gene was visible in the lane with reverse transcribed MCF7 mRNA + β-estradiol confirming it was chimeric. In all cases, gene amplification was not visible in the negative control lanes (MCF7 mRNA without reverse transcriptase: Fig. 5, lane 3 of each set). These data prove that the cDNAs of GREB1a, GREB1b and GREB1c represent the expression of different mRNAs with divergent 5′ untranslated exons.

GREB1 is a primary target for estrogen receptor

In order to determine if GREB1 and PDZK1 are primary targets for estrogen receptor regulation, MCF7 cells were exposed to β-estradiol in the presence or absence of cycloheximide. As seen in Figure 6, GREB1 is expressed in the presence of cycloheximide. The slight increase over estradiol alone is likely the result of stabilization of mRNA known to occur with cycloheximide treatment. PDZK1, however, is repressed by cycloheximide indicating the need for new protein synthesis for expression. This finding demonstrates that PDZK1 is a secondary target for estradiol induction and the PDZK1 promoter is not likely to be dependent on an ERE. The pS2 gene is expressed in the presence of cycloheximide, although the level of expression is slightly reduced. This result suggests that there may be additional mechanisms increasing expression of pS2 that require synthesis of new protein. The result with actin confirms the abundance of mRNA in each lane.

Expression in primary breast cancers

In order to understand the biological significance of the estrogen induced breast cancer genes isolated in this study, the expression of GREB1 and PDZK1 were evaluated in primary breast cancers by RT-PCR. Primers for this experiment were designed using sequence information from the cloned GREB1a and from the published sequence of PDZK1 (GenBank Ac# NM_002614).

Preliminary experiments were performed in the absence of reverse transcriptase to verify that the PCR amplification was due to reverse transcription of mRNA present in the tumor samples and not due to contamination with genomic DNA in the total RNA extract. Qualitative analysis of the expression of the three genes is presented in Figure 7a. As the figure indicates, GREB1 and PDZK1 were predominantly present in ER-positive breast tumors with only a few of ER-negative tumors exhibiting gene expression above background levels. Semi-quantitative analysis was performed by determining signal intensity using phosphorimaging. Values were normalized against β-actin to determine relative expression of the different genes analyzed in this study. As indicated in Figure 7b, GREB1 expression was 3.5-fold greater in ER-positive compared to ER-negative breast tumors. Similarly PDZK1 gene expression was 19-fold greater in ER-positive breast tumors than in ER-negative breast tumors.

DISCUSSION

Estrogen has profound effects on the growth and differentiation of hormone responsive tissues (29, 30). In ER-positive breast cancer, estrogen acts as a mitogen and anti-estrogens such as tamoxifen are the main form of chemotherapy (31-33). Despite the importance of estrogen function, our understanding of the molecular details controlling physiologic responses to estrogen is incomplete. The pS2 gene is one of the most extensively studied of the estrogen-responsive genes (17). However, pS2 is not expressed in all ER-positive breast carcinoma cell lines (see Fig. 3) and is only expressed in 30-60% of ER-positive tumors (21-23). In addition, pS2 null/null mice have hyperplasia of gastric mucosa but do not appear to have altered responses to estrogen (34). More recently, attention has been focused on cyclin D1 as an important mediator of the response of tumors to estrogen (35). Progesterone receptor (PR) (9), HSP27 (19) and TGFβ (36), are other genes induced by estrogen in hormone-responsive breast cancer that have been extensively studied. Although several physiologic functions have been ascribed to these genes, the expression of the known estradiol-regulated genes fails to adequately explain the effects of estrogen in hormone-responsive tumors. For this reason, we have undertaken a study to identify additional genes induced in hormone-responsive tumors in response to estradiol treatment. Specifically, we were interested in identifying genes that were induced by estradiol, repressed by tamoxifen and that demonstrated a high degree of correlation with ER expression in breast cancer cell lines and primary tumors. Two genes were identified—PDZK1 and GREB1—that fulfill these criteria.

Several methods have been described to identify estrogen-regulated genes. Early research studying estrogen-induced gene expression employed techniques such as double isotope protein labeling of induced proteins followed by analysis on SDS polyacrylamide gel electrophoresis (14). The pS2 gene had been identified using the technique of differential library screening (17). This technique is useful for identifying abundant genes but does not allow isolation of scarce mRNAs (15). SSH has been recently described for

the identification of differentially expressed genes and has several advantages over older methods (24). SSH allows isolation of differentially expressed mRNAs of low abundance with a false positive rate of 30-80% (15, 37). SSH has not previously been applied to the isolation of estradiol-induced genes and earlier studies suggested that there are many more estradiol-regulated genes than had previously been reported (15). For these reasons, applying this new technique to identify estradiol-responsive genes was likely to result in the isolation of genes critical to hormone response.

Four of the estrogen-induced genes identified in this study were genes that had been reported previously. These genes include thombospondin, PDZK1, an mRNA coding for an unidentified protein (KIAA0575) from human brain (herein termed GREB2) and an immunoglobulin-like gene. None of these had previously been identified as being responsive to estradiol. Thrombospondin is a matrix-bound adhesive glycoprotein, which is present in a variety of cells, including mammary epithelial cells and osteoblasts (38). It has been shown to play a role in tumor angiogenesis and tumor cell proliferation (39, 40). A study of human osteoblast-like cells exhibited co-localization of thrombospondin with transforming growth factor beta-I (TGF beta), and insulin-like growth factor-I (IGF-I). These proteins were expressed in the extracellular matrix and were modulated by β-estradiol (41). However, in another study, thrombospondin was not found to be responsive to estrogen in ER-positive MCF7 breast carcinoma cells (40). Our study established that although thrombospondin did not correlate with ER in a panel of breast carcinoma cell lines, there was approximately a 4-fold upregulation of expression in MCF7 cells in response to β-estradiol treatment.

One of the genes induced by estrogen identified in these studies was PDZK1. The PDZ domain was named for three proteins in which the domain was first recognized—post-synaptic density protein PSD-95 (42), Drosophila disc large tumor suppressor *dlg* (43) and the tight junction-associated protein ZO-1 (44). Proteins containing this domain have been reported to be involved in organizing proteins at the cell membrane (45) and are also

involved in linking transmembrane proteins to the actin cytoskeleton (46). Through these interactions, PDZ domain proteins regulate a diverse set of cell functions including control of signal transduction, determining cell polarity, cell differentiation (47) and ion transport (48). PDZK1 was first isolated in a yeast two-hybrid screen designed to identify proteins interacting with MAP17—a membrane-associated protein involved in regulation of cell proliferation (49). Subsequently, PDZK1 has been reported to interact with cMOAT(MRP2) which is a canalicular organic anion transporter associated with multidrug resistance (50). Expression of PDZK1 is limited to epithelial cells and over-expression of this protein has been reported in a variety of carcinomas (49). PDZK1 contains four PDZ domains and could interact with a number of proteins simultaneously thereby coordinating the interplay of multiple proteins at the cell membrane. The demonstration that PDZK1 is induced by estradiol in hormone-responsive breast cancer provides an important mechanism to explain membrane alterations such as formation of microvilli that occurs with estrogen treatment (51). It has recently been reported that estrogen induces expression of NHE-RF—the human homolog of the Na+/K+ exchange regulatory factor (52). These results provide important clues related to the physiologic effects of estrogen on cell membrane alterations.

The GREB1 gene demonstrated a striking correlation with ER expression in a panel of breast cancer cell lines. Expression of GREB1 was similarly associated with ER expression in primary breast cancers, although the correlation was not perfect. However, cancer specimens obtained from patients are not controlled for ligand exposure. Since expression of these genes is dependent on estrogen, it might be expected that tumors resected from post-menopausal women might not demonstrate expression of estrogen-induced genes. In addition, the use of estrogens and newer anti-estrogens such as raloxifene may alter patterns of gene expression. However, these results do establish that these estrogen-regulated genes are expressed in primary breast tumors.

The structure of GREB1 is complex in that there are at least three different non-coding 5′ exons. The expression of each of these transcripts is estrogen-regulated suggesting the presence of multiple estrogen-inducible promoters. The occurrence of multiple estrogen-inducible promoters is not unique. The PR gene has been shown to have multiple estrogen-regulated promoters that are controlled by distinct EREs. In the case of PR these different transcripts encode two distinct PRs, PR A and B, which have different transcriptional activation ability (53). The different 5′ exons for GREB1 are non-coding and each transcript utilizes the same initiation codon. However, there are various splicing patterns involving the 3′ end of the gene that could result in proteins with different carboxyl termini. Recently the mouse homolog of this gene was identified but no function has been determined (54). Although the function of this gene remains unknown, the pattern of expression and regulation by estrogen implies an important function in hormone-responsive breast cancer.

In conclusion, SSH was used to identify estradiol-regulated genes in hormone-responsive breast cancer. Of the fourteen genes identified, thirteen were induced early (6 hours) and all were repressed with tamoxifen. In addition, SSH was used to compare the pattern of gene expression in the absence of estrogen and in the presence of estrogen together with tamoxifen. No significant differences were detected suggesting that tamoxifen is a pure anti-estrogen in breast tissue. These results indicate that the only effect that tamoxifen has on gene expression occurs through its interaction with ER. Two estrogen-regulated genes were identified—PDZK1 and GREB1. The pattern of expression of these genes suggests an important role of these proteins in the physiologic response of tumors to estrogen.

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Figure legends (Ghosh, et al.)

Figure 1: Northern blots of Genes Regulated by Estrogen in Breast cancer (GREB) identified by SSH. Northern blots containing 1μg of poly(A)+RNA per lane from MCF\-estradiol, MCF7\+estradiol and MCF7\+estradiol+tamoxifen were probed with cDNA fragments of genes isolated by SSH. A representitive Northern blot for each estrogen-responsive gene identified is shown. Several genes were detected multiple times and are indicated. In addition, pS2 is shown as a control to validate estradiol responsiveness. ER, GAPDH and β-actin were used as size markers and the latter two also confirm similar loading of the samples.

Figure 2: Kinetics of Estradiol Response. Northern blots containing 1μg of poly(A)+RNA per lane from MCF7 cells grown in presence and absence of estradiol for 6, 24 or 48 hours. As shown, GREB1, GREB2 and PDZK1 respond early to estradiol treatment. The known estradiol responsive gene, pS2 is shown as a control. Hybridization with β-actin was used to confirm approximately equal loading of poly(A)+RNA .

Figure 3: Expression of GREB1 and PDZK1 in breast cancer cell lines. Northern blots demonstrating the gene expression pattern of GREB1 and PDZK1 compared to pS2 and ER in a panel of breast carcinoma cell lines. Lanes 1-5 are cell lines that express high levels of ER (MCF7, T-47D, MDA-MB-361, ZR-75-1 and BT-474), lane 6, (BT-20), is a cell line that expresses very low levels of ER, and lanes 7-8 are ER-negative cell lines (MDA-MB-231, and HBL-100). GAPDH was used as a control to confirm similar loading of poly(A)+RNA .

Figure 4: Structure, Nucleotide and Amino Acid sequence for GREB1 transcripts (A) Schematic diagram of GREB1a cDNA determined from cDNA cloning,

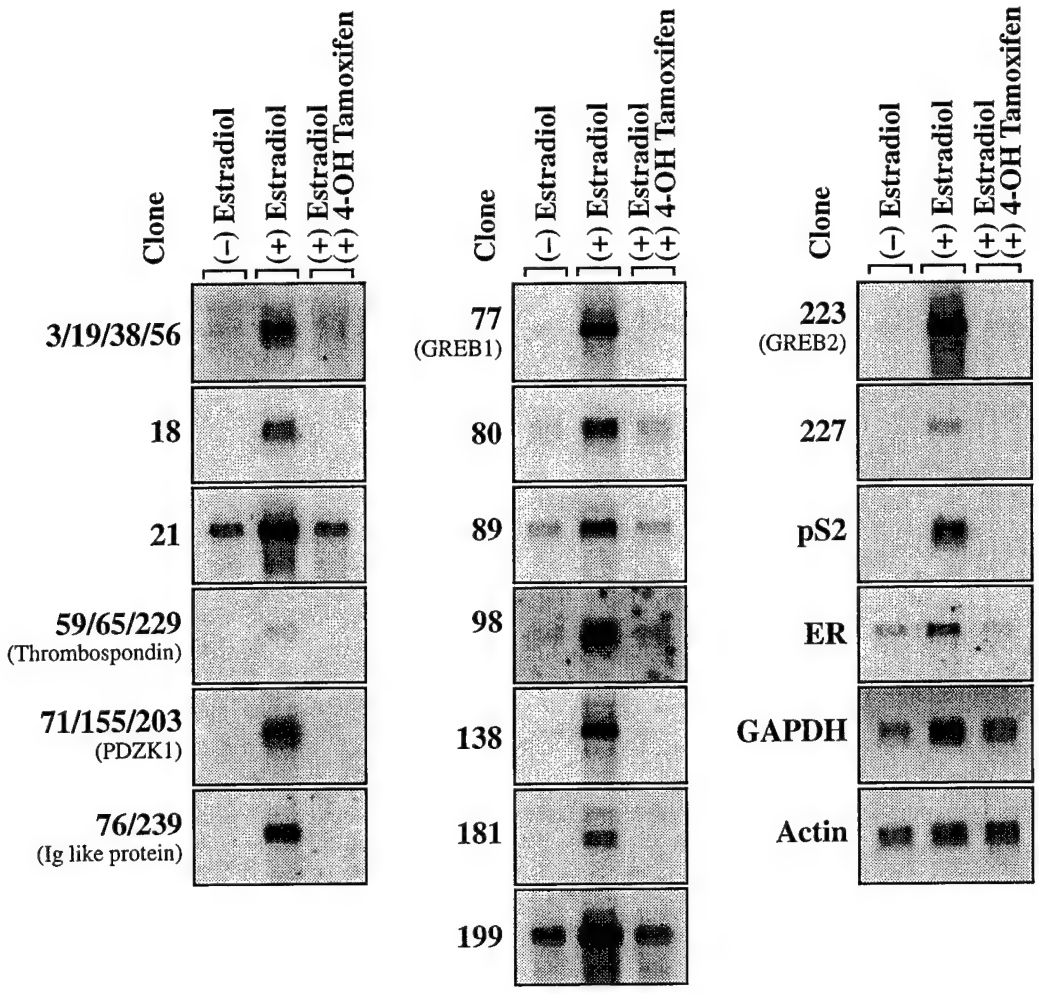
RT-PCR and and published sequence of AB011147. Predicted protein of longest clone is 1949 aa. Below is schematic of GREB1a, GREB1b and GREB1c transcripts cDNAs obtained from library screening. **(B)** Predicted amino acid sequence of GREB1a protein. Using DNA Strider 1.2 (28), the GREB1a transcript is predicted to have 300 bp 5′ untranslated region (UTR) preceding the initiation site of the putative open reading frame (ORF). Sequence homology between GREB1a and GREB1b and between GREB1a and GREB1c is indicated using the symbols (✤) and (♦), respectively. Within the homologous region of the three transcripts, GREB1c contains a single nucleotide change from GREB1a and GREB1b the location of which is indicated with (•). **(C)** The nucleotide sequences of the different 5′ and 3′ ends for various transcripts of GREB1 are shown. The sequences of the alternate 3′ ends of GREB1b and GREB1c with the putative ORFs of GREB1b and GREB1c continues for an additional 28 and 71 bps respectively, beyond the sequence homologous with GREB1a. A 729 bp 3′ UTR was present in the GREB1b transcript while the GREB1c transcript contained a 790 bp 3′ UTR ending with a poly A tail. For alternate 5′ end of GREB1, the beginning of sequence identity between GREB1a and GREB1c is indicated with (↑). Although other transcripts also begin sequence homology at (↑), the GREB1b contained 13 bp deletion prior to the region of sequence homology with other transcripts indicated with (⊥).

Figure 5: Verification of differential splicing of GREB1. RT-PCR was used to verify the structure of the different GREB1 transcripts isolated from the MCF7 cDNA library. For each transcript, four sets of RT-PCR reactions were performed. A 5′ primer common to all transcripts and unique 3′ primers that were designed against unique regions for each of GREB1a, GREB1b, GREB1c and GREB1d. Templates for the 4 reactions are as follows: cDNA control, reverse transcribed poly(A)+RNA , poly(A)+RNA without reverse transcriptase and zero template.

Figure 6: GREB1 is a primary target for estrogen receptor. Expression of GREB1 and PDZK1 was examined following exposure to estrogen in the presence or absence of cycloheximide. MCF7 cells were grown in the absence of β-estradiol, or in the presence of β-estradiol for 24 hours without or with cycloheximide. Northern blots were probed with GREB1, PDZK1, pS2 or β-actin.

Figure 7: Examination of the expression GREB1 and PDZK1 in primary breast tumors. (A) Expression of GREB1, PDZK1, ER and β-actin was examined by RT-PCR in immunohistochemically determined ER-positive, ER-negative breast tumors and normal breast tissue. Reverse transcribed Poly(A)+RNA from MCF7(+E) served as the positive control, while that from MCF7(-E) and MDA-MB-231 and zero template were negative controls. Following PCR 10 μl of each sample was electrophoresed on 1.5% agarorse, Southern blotted and probed with internal probes that were radioactively endlabeled with ^{32}P. **(B)** Expression of estrogen-responsive genes in ER-positive versus ER-negative breast tumor samples. Signal intensity from southern blots was quantified using phosphor imaging system of Molecular Dynamics, Sunnyvale, CA. Semi-quantitative values were obtained by normalizing signals against β-actin to determine relative expression of GREB1 and PDZK1.

FIGURE 1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(+) Estradiol
(+) Estradiol
(+) 4-OH Tamoxifen
3/19/38/56
18
21
59/65/229
(Thrombospondin)
71/155/203
(PDZK1)
76/239
(Ig like protein)
Clone
(–) Estradiol
(+) Estradiol
(+) Estradiol
(+) 4-OH Tamoxifen
77
(GREB1)
80
89
98
138
181
199
Clone
(–) Estradiol
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(+) 4-OH Tamoxifen
223
(GREB2)
227
pS2
ER
GAPDH
Actin

FIGURE 2

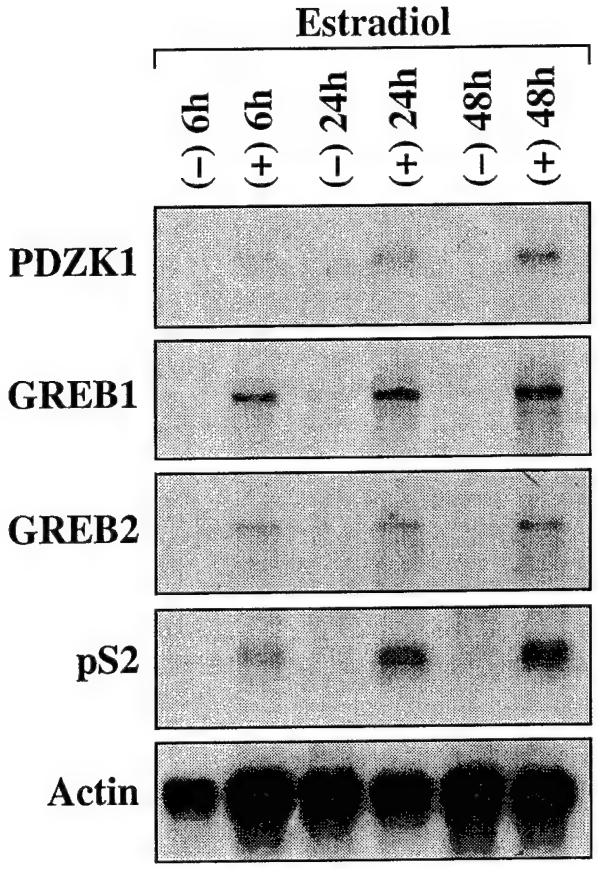
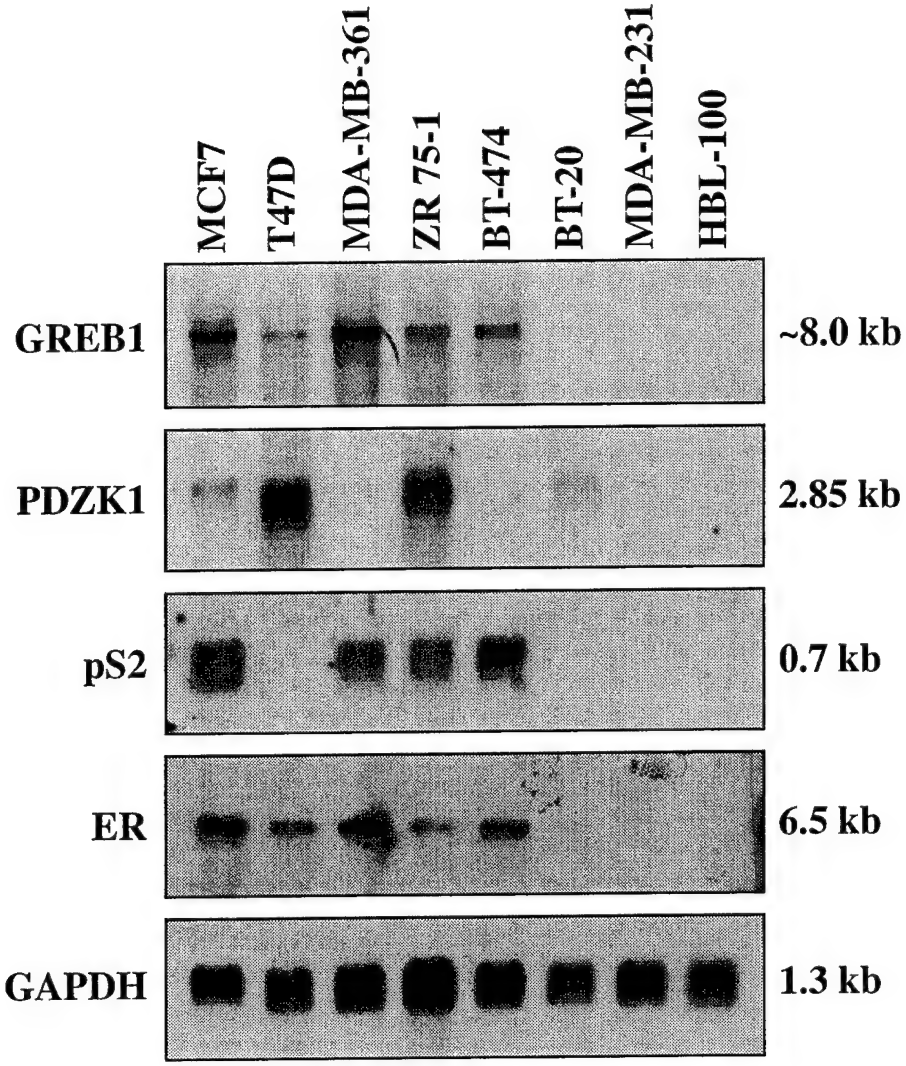


FIGURE 3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ZR 75-1
BT-474
BT-20
MDA-MB-231
HBL-100
GREB1
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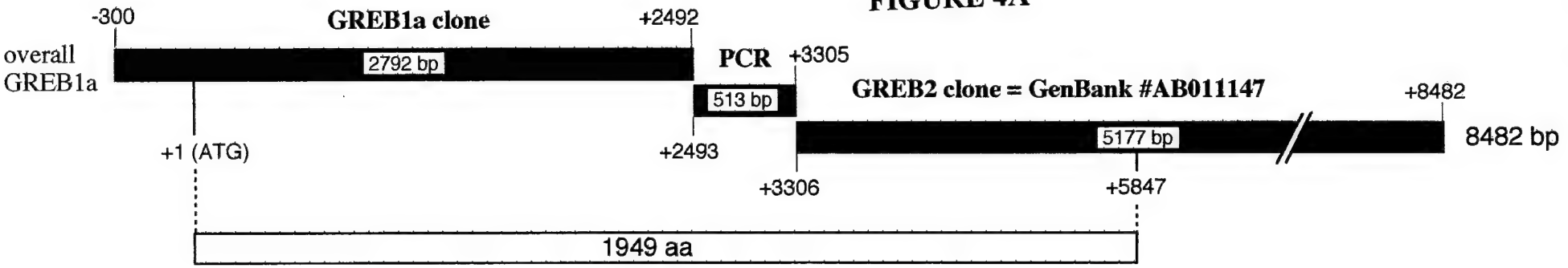
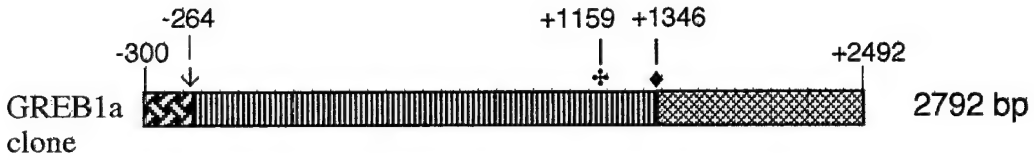
PDZK1
2.85 kb
pS2
0.7 kb
ER
6.5 kb
GAPDH
1.3 kb

FIGURE 4A


-300
GREB1a clone
+2492
overall GREB1a
2792 bp
PCR
+3305
513 bp
GREB2 clone = GenBank #AB011147
+8482
+1 (ATG)
+2493
5177 bp
8482 bp
+3306
+5847
1949 aa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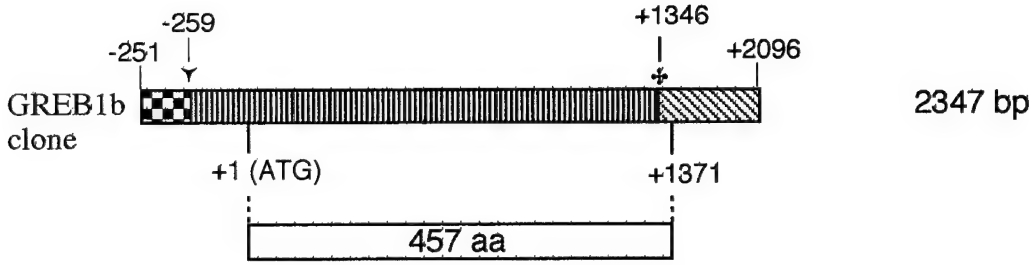
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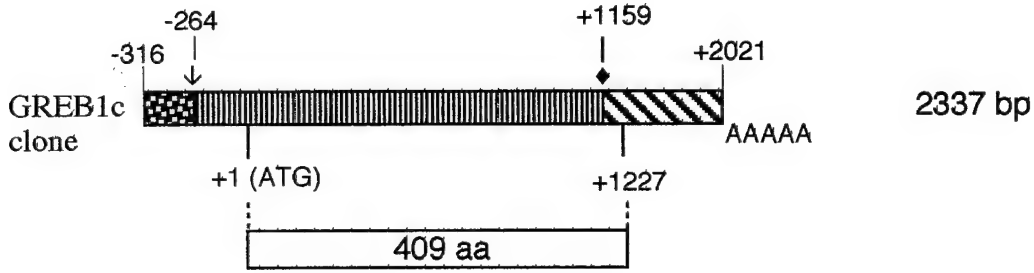
457 aa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+1 (ATG)
+1227
409 aa

FIGURE 4B

Sequence of GREB1a protein

```
   1  MGNSYAGQLK TTRFEEVLHN SIEASLRSNN LVPRPIFSQL YLEAEQQLAA LEGGSRVDNE EEEEEGEGGL ETNGPPNPFQ LHPLPEGCCT TDGFCQAGKD  100
 101  LRLVSISNEP MDVPAGFLLV GVKSPSLPDH LLVCAVDKRF LPDDNGHNAL LGFSGNCVGC GKKGFCYFTE FSNHINLKLT TQPKKQKHLK YYLVRNAQGT  200
 201  LTKGPLICWK GSEFRSRQIP ASTCSSSLFP ALESTAAFPS EPVPGTNPSI LMGAQQAGPA SDHPSLNAAM GPAVFNGKDS PKCQQLAKNN LLALPRPSAL  300
 301  GILSNSGPPK KRHKGWSPES PSAPDGGCPQ GGGNRAKYES AGMSCVPQVG LVGPASVTFP VVASGEPVSV PDNLLKICKA KPVIFKGHGN FPYLCGNLND  400
 401  VVVSPLLYTC YQNSQSVSRA YEQYGASAIQ PISEEMQLLL TVYYLVQLAA DQVPLMEDLE QIFLRSWRES HLTEIRQYQQ APPQPFPPAP SAAAPVTSAQ  500
 501  LPWLASLAAS SCNDSVHVIE CAYSLAEGLS EMFRLLVEGK LAKTNYVVII CACRSAAIDS CIAVTGKYQA RILSESLLTP AEYQKEVNYE LVTGKVDSLG  600
 601  AFFSTLCPEG DIDILLDKFH QENQGHISSS LAASSVTKAA SLDVSGTPVC TSYNLEPHSI RPFQLAVAQK LLSHVCSIAD SSTQNLDLGS FEKVDFLICI  700
 701  PPSEVTYQQT LLHVWHSGVL LELGLKKEHM TKQRVEQYVL KLDTEAQTKF KAFLQNSFQN PHTLFVLIHD HAHWDLVSST VHNLYSQSDP SVGLVDRLLN  800
 801  CREVKEAPNI VTLHVTSFPY ALQTQHTLSS PYNEIHWPAS CSNGVDLYHE NKKYFGLSEF IESTLSGHSL PLLRYDSSFE AMVTALGKRF PRLHSAVIRT  900
 901  FVLVQHYAAA LMAVSGLPQM KNYTSVETLE ITQNLLNSPK QCPCGHGLMV LLRVPCSPLA VVAYERLAHV RARLALEEHF EIILGSPSSG VTVGKHFVKQ 1000
1001  LRMWQKIEDV EWRPQTYLEL EGLPCILIFS GMDPHGESLP RSLRYCDLRL INSSCLVRTA LEQELGLAAY FVSNEVPLEK GARNEALESD AEKLSSTDNE 1100
1101  DEELGTEGST SEKRSPMKRE RSRSHDSASS SLSSKASGSA LGGESSAQPT ALPQGEHARS PQPRGPAEEG RAPGEKQRPR ASQGPPSAIS RHSPGPTPQP 1200
1201  DCSLRTGQRS VQVSVTSSCS QLSSSSGSSS SSVAPAAGTW VLQASQCSLT KACRQPPIVF LPKLVYDMVV STDSSGLPKA ASLLPSPSVM WASSFRPLLS 1300
1301  KTMTSTEQSL YYRQWTVPRP SHMDYGNRAE GRVDGFHPRR LLLSGPPQIG KTGAYLQFLS VLSRMLVRLT EVDVYDEEEI NINLREESDW HYLQLSDPWP 1400
1401  DLELFKKLPF DYIIHDPKYE DASLICSHYQ GIKSEDRGMS RKPEDLYVRR QTARMRLSKY AAYNTYHHCE QCHQYMGFHP RYQLYESTLH AFAFSYSMLG 1500
1501  EEIQLHFIIP KSKEHHFVFS QPGGQLESMR LPLVTDKSHE YIKSPTFTPT TGRHEHGLFN LYHAMDGASH LHVLVVKEYE MAIYKKYWPN HIMLVLPSIF 1600
1601  NSAGVGAAHF LIKELSYHNL ELERNRQEEL GIKPQDIWPF IVISDDSCVM WNVVDVNSAG ERSREFSWSE RNVSLKHIMQ HIEAAPDIMH YALLGLRKWS 1700
1701  SKTRASEVQE PFSRCHVHNF IILNVDLTQN VQYNQNRFLC DDVDFNLRVH SAGLLLCRFN RFSVMKKQIV VGGHRSFHIT SKVSDNSAAV VPAQYICAPD 1800
1801  SKHTFLAAPA QLLLEKFLQH HSHLFFPLSL KNHDHPVLSV DCYLNLGSQI SVCYVSSRPH SLNISCSDLL FSGLLLYLCD SFVGASFLKK FHFLKGATLC 1900
1901  VICQDRSSLR QTVVRLELED EWQFRLRDEF QTANAREDRP LFFLTGRHIZ                                                        1949
```

FIGURE 4C

3' end of GREB1b protein

```
1345  GCA CAT CAA ATA CGA AAT CCG GAC GTA TAA  1374
 449   A   H   Q   I   R   N   P   D   V   Z   457
       ÷
```

3' end of GREB1c protein

```
1156  AAA GTA TTT GTA AAT GGT GCT ACC CAA ATG GTA GCC CTT GGT CCG GCA GAA CCT GCA TCG CCC CGG AGC TTA TGA  1230
 386   K   V   F   V   N   G   A   T   Q   M   V   A   L   G   P   A   E   P   A   S   P   R   S   L   Z    409
       •
```

alternate 5' ends of GREB

5'-AAATAAAGTTTTTTCAATGGAAGGCTTGCAGCTCTTGAGGACCTGCCAAATGGAAGAAGGACAGAGACCTGGAGCCCTATGGAAAGTTCTGACACCATGTGTGGAAGGACATGGCTTTTAACACGTGTGGTGACTGG**AGTAGCTG**-3' GREB1a

5'-GAAAGAATCAAAGCGCACATCTTGTACTTTGATGCCCATAGGAAGGGCTCTCCTCTGGCCCCTCTGGCTTTGTTTGGAGCAGAAAACAACAAA-------------**CTG**-3' GREB1b

5'-GAAAGAATCAAAGCGCACATCTTGTACTTTGATGCCCATAGGAAGGGCTCTCCTCTGGCCCCTCTGGCTTTGTTTGGAGCAGAAAACAACAAAGTGAGTTC**AGTAGCTG**-3' GREB1d

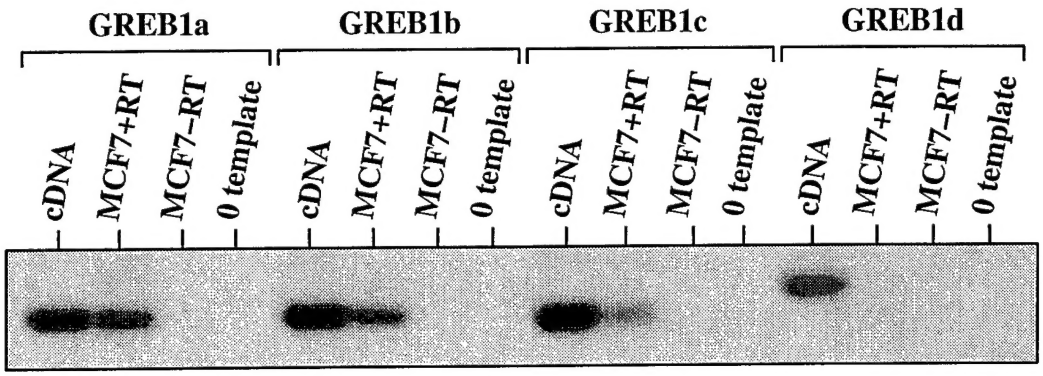
5'-GAAAGAATCAAAGCGCACATCTTGTACTTTGATGCCCATAGGAAGGGCTCTCCTCTGGCCCCTCTGGCTTTGTTTGGAGCAGAAAACAACAAAGTGAGTTC**AGTAGCTG**-3' GREB1b(i)

5'-GAGCAGCAGTGTGTTCATGGAGAAAGAATCAAAGCGCACATCTTGTACTTTGATGCCCATAGGAAGGGCTCTCCTCTGGCCCCTCTGGCTTTGTTTGGAGCAGAAAACAACAAAGTGAGTTC**AGTAGCTG**-3' GREB1b(ii)

5'-GCATTACGCGCCCCACGCATCCTCTTCCATCCCCAGGCACAGATCAAAGGCGCAGCCCAGGAGGCGGGAGCCCCTGCACACTTTCCACCTCTGCTGGGCTTAGCCTCTTGGCTGGTTGGTCTGTGGAGTGCCTGAAGTGACCAGCTTTTTGTA**AGTAGCTG**-3' GREB1c

5'-GTTACTTATATAACTTCCTGTAATTGCCCGGC**AGTAGCTG**-3' GREB1e

FIGURE 5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MCF7–RT
0 template
cDNA
MCF7+RT
MCF7–RT
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FIGURE 6

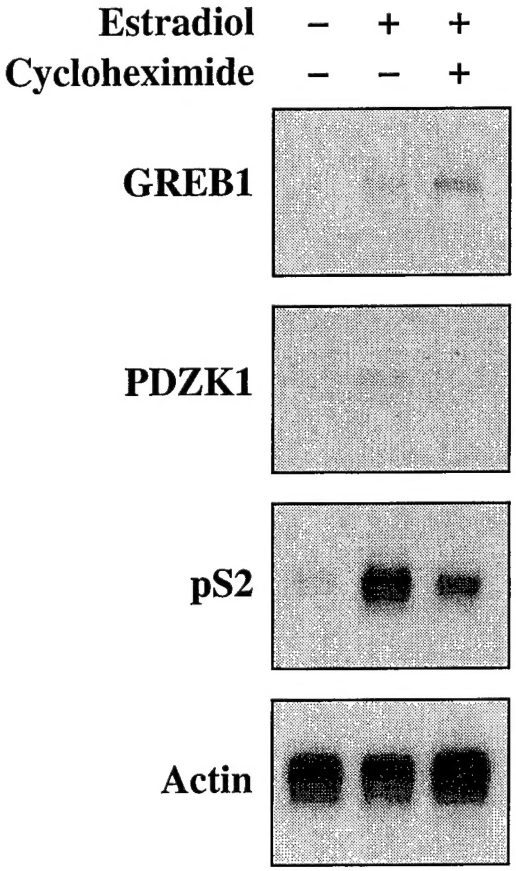

FIGURE 7A

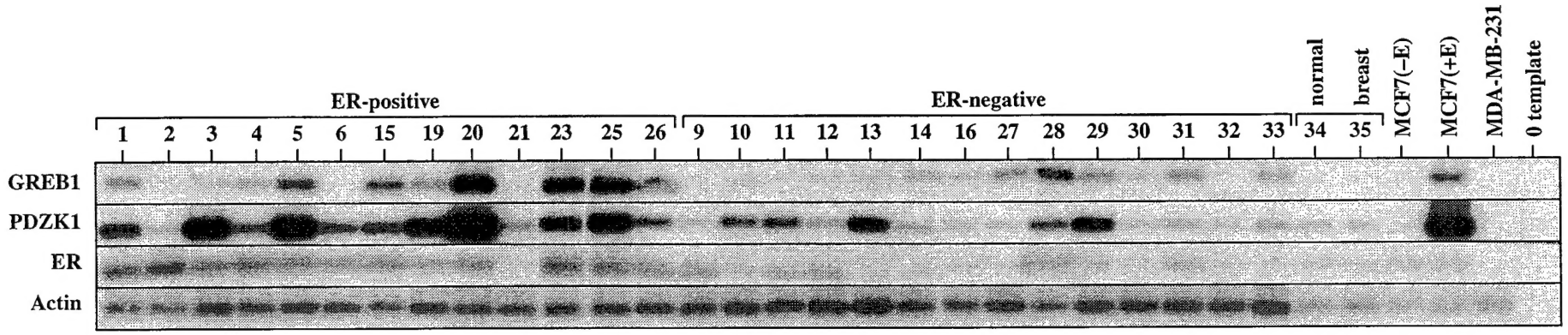

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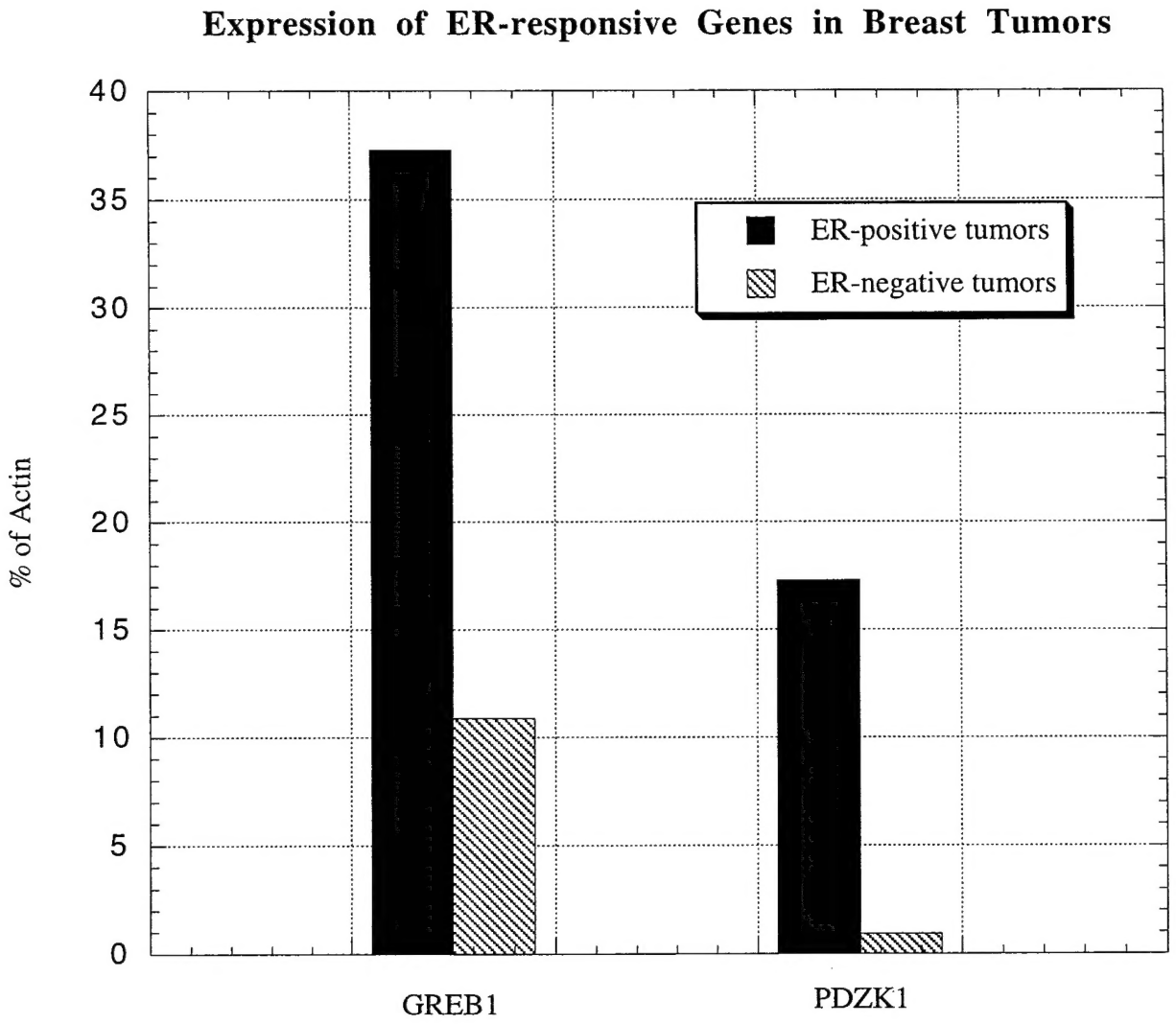
FIGURE 7B


Expression of ER-responsive Genes in Breast Tumors
% of Actin
40
35
30
25
20
15
10
5
0
ER-positive tumors
ER-negative tumors
GREB1
PDZK1